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Attenuation of Cellular Inflammation Using Glucocorticoid-Functionalized Copolymers

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Abstract— **This work has demonstrated the functionalization of an amphiphilic diblock copolymer, comprised of polyethylene oxide-polymethyl methacrylate (PEO-PMMA), as well as a triblock copolymer comprised of polymethyloxazoline–polydimethylsiloxane-polymethyloxazoline(PMOXA–PDMS-PMOXA) with the dexamethasone (Dex) glucocorticoid anti-inflammatory. Interfacial deposition of the copolymer and the Dex molecules and subsequent transfer of the hybrid materials to solid substrates were characterized to evaluate the potential of utilizing this composite material as a suppressor of cyto-inflammation to enhance implant biocompatibility. Given the extremely thin dimensions of the film (~4nm), this material would have negligible impact upon the size of the coated device to preclude biological stress. The composite films were interfaced with the RAW264.7 murine macrophages which served as a model cell line for the evaluation of Nuclear factor-kappaB (NF-κB)-induced production of a host of inflammatory cytokines including interleukin-6, interleukin-12, tumor necrosis factor-alpha (TNFα), as well as the inducible Nitric Oxide Synthase signaling factor which is known to be involved with stress-related processes such as neuronal damage. Lipopolysaccharide or LPS is a component of bacterial membranes that elicits cellular stress following application to RAW cell cultures. Following the induced stress response, significant reductions in the expression of genes associated with the aforementioned cytokines and signaling molecules indicated that macrophages in direct contact with the functionalized copolymer were able to collect Dex that was released from within the polymer network to attenuate cyto-inflammation mechanisms. This composite membrane represents a medically-relevant technology to promote chronic implant functionality and preclusion of bio-fouling.**

Index Terms—**Cell regulation, copolymer, biomaterials**

I. Introduction

Block copolymers have been shown to be effective matrices to support protein function for the mimicry of key natural biological processes such as energy conversion, as well as voltage-gated ion transport [1-9]. While conventional lipid-based systems have enabled single protein characterization and mechanisms of functionality to be elucidated [10-13], block copolymers represent a highly versatile approach towards tailored biology, whereby specific properties can be engineered into the material to accommodate specific protein geometries, desired block lengths, compositions, and charge properties, to name a few. In addition, the addition of endgroups, such as acrylate, can be made to enable UV, or chemically-induced polymeric crosslinking to enhance material stability for enhanced device robustness.

Block copolymers, such as the PEO-PMMA and PMOXA-PDMS-PMOXA materials that were utilized for this study can also possess the amphiphilic properties (polymer structure has alternating hydrophilic and hydrophobic properties) that enable material integration at the air-water interface and subsequent deposition onto solid substrates via the Langmuir-Blodgett modality. More specifically, this technique can be used to deposit large area, uniform thin films to coat solid state devices, such as those used for implants. Deposition of the amphiphilic molecules is carried out by adding the polymer to a subphase of water, and due to the alternating hydrophobic-hydrophilic nature of the amphiphile, polymeric solubilization into the subphase is then precluded. Compression of the interfacial material then enables stable film formation.

We have demonstrated the deposition of the PEO-PMMA and PMOXA-PDMS-PMOXA copolymers at the air-water interface followed by the lacing of the copolymers with the Dex anti-inflammatory for subsequent composite film transfer to the substrate. RAW264.7 culture atop these functionalized substrates revealed substantially decreased LPS-induced cellular inflammation which was indicative of polymer-mediated transfer of Dex across the cell membrane to suppress NF-κB-induced stress response. Langmuir isotherms were utilized to confirm the maintenance of the hydrophilic Dex at the air-water interface while quantitative real time Polymerase Chain Reaction (RT-PCR) was utilized to assess gene program attenuation by quantifying inflammatory gene mRNA expression (Fig. 1).

II. Materials and Methods

A. Polymer Preparation and Characterization

The PMOXA-PDMS-PMOXA and PEO-PMMA copolymers were solubilized to 0.1mg/ml in chloroform and stirred overnight to result in a translucent, homogeneous solution for LB deposition. We have previously characterized the thickness of the materials, using the PMOXA-PDMS-PMOXA structure as an example, by

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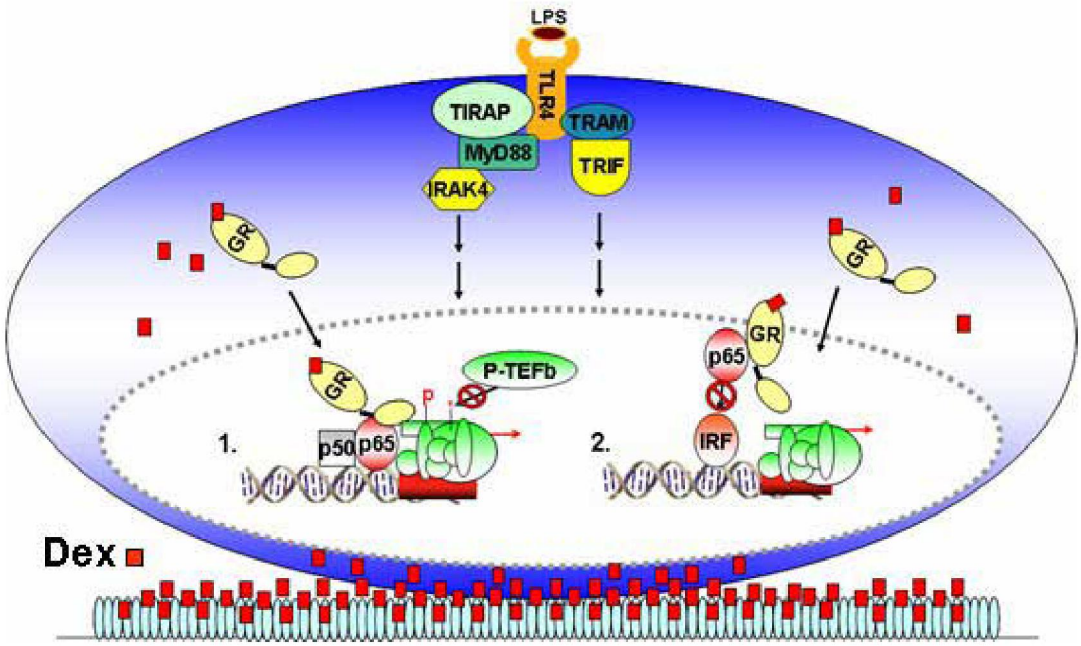

Figure 1. Dex is known to interfere with p65 interaction with transcriptional machinery (e.g. P-TEFb) or coactivators to suppress inflammation.

performing a secondary treatment of the chloroform-solubilized copolymer with toluene and suspending a droplet across a 25μm-thick hydrophobic septum flanked by 2 Ag/AgCl electrodes for capacitance measurements as given by: $C=\varepsilon_o\varepsilon_1 A/d$, where C is the membrane capacitance, ε_0 is the permittivity of free space (=8.9 × 10−19 F/m), ε_1 is the relative dielectric constant of the PDMS hydrophobic block, A is the measured area of the annulus in the septum (=250 μm), and d is the thickness of the membrane. The resultant thickness of the materials were determined to range between 3-4nm [14-18].

B. General Langmuir-Blodgett Film Fabrication Protocol for Polymeric Substrates

LB films were fabricated using a KSV 2000 Standard Langmuir Trough with a Teflon® base and a subphase of water. To preclude sample contamination from ambient particles, the entire trough was covered with a plastic case and a small door was integrated to allow for manual manipulation/cleaning of the trough. The base was cleaned with chloroform using a cotton swab and large tweezers and then rinsed thoroughly with nanopure water. The water was then swept using cotton swabs into the central reservoir of the trough and suctioned off using a vacuum pump. This step was performed three times to ensure trough cleanliness. The trough was then filled with nanopure water while paying careful attention not to deposit water droplets along the edge of the trough. The Wilhemy platinum pressure sensing plate (stored in MeOH was then thoroughly rinsed using nanopure water, and subsequently sterilized using a torch. The pressure sensor was then zeroed and the ready for film deposition (Fig. 2).

C. Glucocorticoid Preparation/Deposition for Inflammation Attenuation Studies

Water soluble dexamethasone (Sigma-Aldrich, Inc.) was dissolved in nanopure water to a concentration of 1mg/ml. The drug was then added to an interfacial pre-formed 10mN/m copolymer film and changes in surface pressure were monitored to confirm dexamethasone presence at the air-water interface. After 30 minutes of allowing the film to reach equilibrium, compressions were performed at a rate of 1mm/min to a maximum pressure of 30mN/m for LB deposition onto glass slides (25mmx75mm) at a rate of 1mm/min (Fig. 3). (VWR Scientific, Inc.) (Films were compressed to >50mN/m until collapse for Langmuir film characterization of film properties). The slides were then used for RAW264.7 murine macrophage culture and quantitative Polymerase Chain Reaction (RT-PCR) analysis.

RAW264.7 (ATCC) cells were cultured at 37°C in DMEM supplemented with 10% FBS and 5% Penicillin/Streptomycin. Following the acquisition of cultures of adequate density, cells cultured on bare glass as well as the composite films were exposed to lipopolysaccharide (LPS) for 4 hours, and slides were subsequently transferred to new Petri dishes and 1ml of TRIzol cell lysis solution was added to wash the slides and collect the genetic material. RNA isolation was done according to the manufacturer's protocol. Subsequent conversion of the RNA to cDNA was performed using the I-script enzyme (Bio-Rad) [19-21].

Following conversion of the isolated mRNA to cDNA, RT-PCR analysis (Bio-Rad, Richmond, CA, USA) was performed to examine the expression of tumor necrosis factor-alpha (TNFα, IP-10, Il-6, and Il-12) primers available upon request) following LPS induction both with and without dexamethasone activity.

III. Results and Discussion

Dexamethasone (Dex) deposition was conducted atop both the PEO-PMMA copolymer and PMOXA-PDMS-PMOXA copolymer structures. Dex presence was confirmed via the application of a FITC-Dex compound atop the pre-formed copolymer monolayer (Fig. 4). FITC-Dex added without the

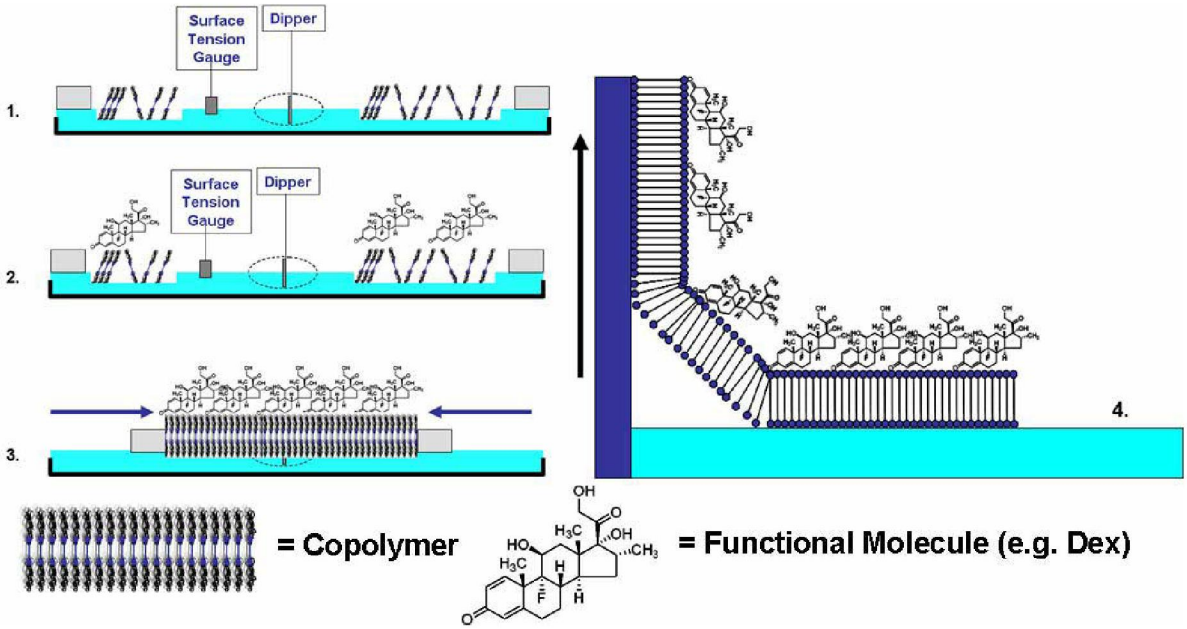

Figure 2. The process for copolymer-mediated Langmuir-Blodgett deposition is shown here. 1) The copolymer was deposited at the air-water interface to a starting pressure of 10-15mN/m. 2) The functional molecule (e.g. Dex) was added to the surface of the pre-formed membrane in a dropwise fashion to minimize copolymer film perturbation. 3) The film was compressed at a rate of 1mm/min to fabricate a uniform, tightly packed composite material that could be tethered to the substrate via the copolymer. 4) The deposition process onto the substrate was carried out at a rate of 1mm/min to ensure complete film formation.

copolymer resulted in the inability to transfer the anti-inflammatory to solid substrates following film compression. Compression isotherms for Dex-only depositions at the air-water interface resulted in the inability to generate a steadily increasing surface pressure isotherm. In fact, the compression isotherm resembled what would commonly be observed if no amphiphile were present at the air-water interface (Data not shown).

Following confirmation of Dex-copolymer composite film fabrication, RAW264.7 cells were cultured atop the active substrates as well as bare glass slides and LPS was utilized to induce cellular stress and the production of a suite of inflammatory cytokines and signaling molecules. LPS binds to the membrane-bound Toll-like receptor 4 (TLR4) that simulates bacterial infection and elicits the activation of transcriptional factors (e.g. Nuclear Factor kappa B (NFκB)) for inflammatory cytokine production. For the purposes of this study, TNFα was
selected as the cytokine marker for macrophage stress, and RT-PCR was utilized to examine the expression of TNFα mRNA (Fig. 5). Macrophages activated by LPS that were cultured atop bare substrates resulted in significantly higher levels of inflammation over samples where LPS was introduced to macrophages that were cultured atop either 3, or 7 layers of Dex-copolymer composites. This outcome demonstrated that the drug-dosing capabilities of the hybrid material could be tailored based upon the number of layers deposited. Because the copolymer membrane dimensions are within the range of 2nm (diblock)-4nm (triblock) in thickness, coating substrates (e.g. implants) with multiple layers would result in minimal impact upon overall device dimensions. In addition, on samples where solutions of Dex were incubated directly with the glass culture slides/self-assembled *without* the polymer, LPS treatment of cultured macrophages resulted in the absence of inflammatory suppression that was previously observed with samples cultured atop the Dex/copolymer composites (Fig. 6). As such, this study showed that 1) Interfacially deposited Dex atop the polymeric amphiphiles could be dispensed to macrophages cultured on the active composite, and 2) Self-assembled Dex without the copolymer substrate was unable to stay tethered to the glass slide for subsequent transfer to the macrophages (Fig. 7).

As demonstrated by the RT-PCR trials, the copolymer served as both a buoying element at the air-water interface for the deposition of Dex, as well as a tethering component to maintain the integrity between the Dex and the glass substrate. This was an expected observation as the nature of the

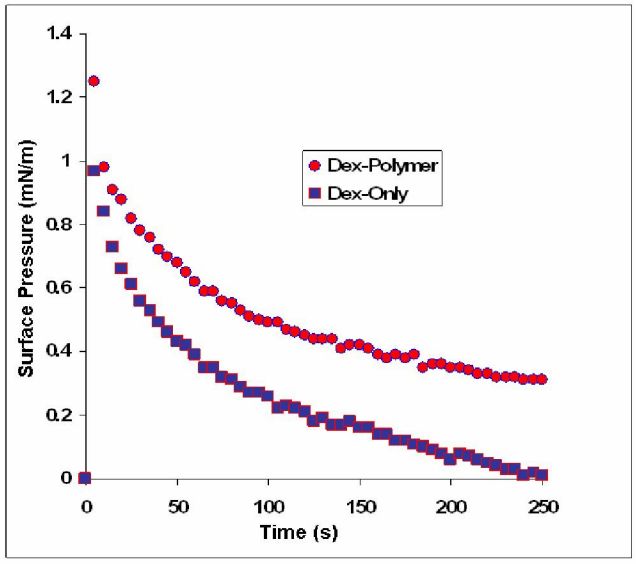

Figure 3. Water-soluble Dex deposited atop the copolymer was suspended at the surface of the Langmuir trough and integrated with the copolymer (circle) while Dex was shown to submerse into the subphase when deposited without the copolymer (square).

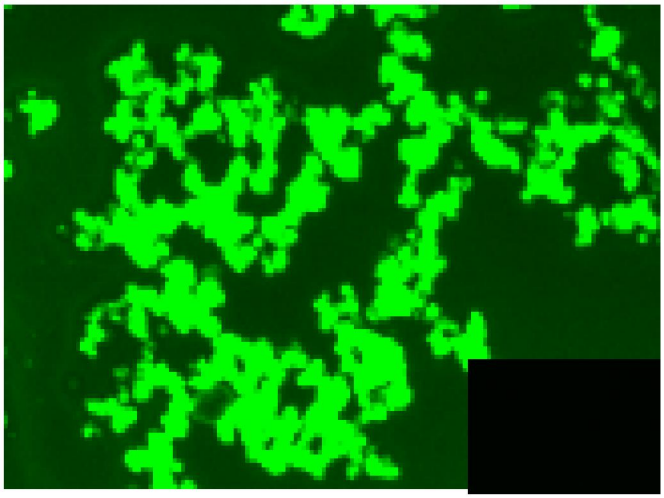

Figure 4. (Top) Dex deposited atop the polymer thin film was shown to be integrated and transferred with the copolymer to the solid substrate for cellular interfacing. (Inset) Dex deposited atop the water was shown using both the isotherm and fluorescence microscopy to submerse into the subphase, precluding transfer onto the substrate.

copolymer-substrate interaction, believed to be based upon a high density Van der Waals interaction, was previously demonstrated. As such, the intercalation of the Dex molecules within these strongly attached PMOXA-PDMS-PMOXA and PEO-PMMA copolymers was expected to be maintained, and potentially enhanced following endgroup crosslinking via UV exposure. This method of treatment to enhance material robustness may not be amenable towards more conventional, lipid-based amphiphiles that are less robust than the copolymer molecules as UV violet wavelengths may irreversibly damage lipid molecule structure. Suppression of LPS-mediated induction of TNFα in RAW264.7 cells demonstrated the ability of these copolymers in suppressing immune response through Dex activation of the glucocorticoid receptor, GR, which results in GR binding and inhibition NF-kB subunit, p65. The activation of GR by Dex has been previously shown to robustly

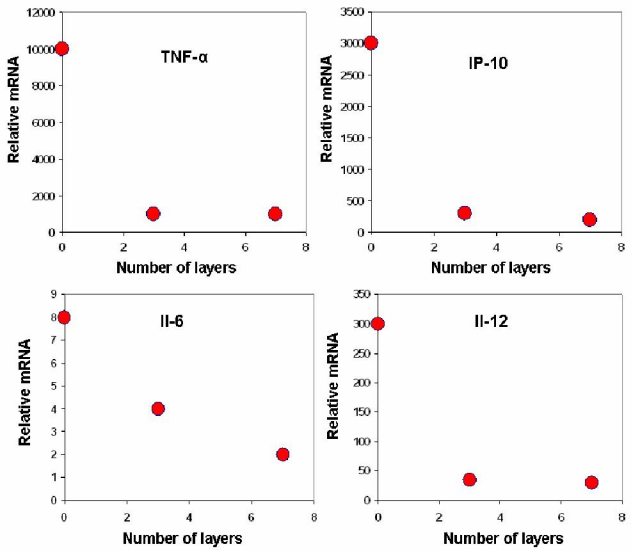

Figure 5. RT-PCR examination TNF, IP-10, Il-6, and Il-12 mRNA show major reductions in inflammation based on the number of DEX- films deposited. These films may be useful towards eliminating initial onset of cellular inflammation that may trigger device fouling.

inhibit NF-kB target genes through the binding of p65 and blocking of p65 recruited transcriptional machinery, such as P-TEFb [22].

With respects to assessing the chronicity of material functionality, continued work will address the replenishing of Dex stores within the planar copolymers that have run out of the corticosteroid. This may be potentially accomplished using vesicular targeting strategies. More specifically, due to the ability to functionalize the surface of copolymer vesicles that are enclosing Dex, planar films that are situated on solid substrates may be interfaced with injectable vesicles for targeted interactions to restore the corticosteroid supplies.

This study has demonstrated the concept of applying copolymer amphiphiles as the foundational element of fabricating thin films with versatile functionalities. Using stress-suppression as an exemplary case, we have shown that the copolymer element enabled functional material (Dex) deposition, and played a secondary role as an anti-protein adsorption and tethering component. As such, this methodology represents a broadly applicable technique for life science studies as well as potential medical applications.

IV. Conclusion

We have demonstrated the fabrication of a robust, copolymer-based therapeutic material for the suppression of cellular stress/inflammation. Following the deposition of single molecule thick copolymer Langmuir films, the copolymers were functionalized with Dex, a corticosteroid known to attenuate NF-κB mediated inflammatory response. Following the interfacing of RAW264.7 murine macrophages with the hybrid therapeutic material, RT-PCR was performed to examine cytokine release from the macrophages. Results showed significant decreases in inflammation using a spectrum of signaling molecules as read-outs, including gene expression levels of Il-6, Il-12, TNFα, IP-10, and iNOS. As it has previously been shown that iNOS expression can be correlated with cellular injury (e.g. neurons), our results show that this nanoscale composite film of 4nm thickness represents a non-invasive approach to coating various surfaces/substrates that may reside at an *in vitro* or *in vivo* interface. This may include the coating of implants to facilitate chronic device functionality as well as preclusion of bio-fouling. These coatings may also be useful for single-cell studies to examine polymeric activation of cellular gene expression pathways for biotic-abiotic interfacing studies (e.g. mechano-sensation, metabolism, etc.). Furthermore, given the versatility of this technology, the polymers may also be assembled into vesicular structures that can serve as reservoirs for therapeutic molecules. Further engineering of the surface functionalization groups of these vesicles will enable intelligent delivery of drugs to specific areas, such as previously coated planar thin films that require drug replenishment, or the ability to target and facilitate on-demand vesicular endocytosis to prevent excessive dosing. As such, the fruition of these materials as well as the potent performance of these composites is expected to serve as a modality for novel medical capabilities, as well as a platform for fundamental cellular studies.

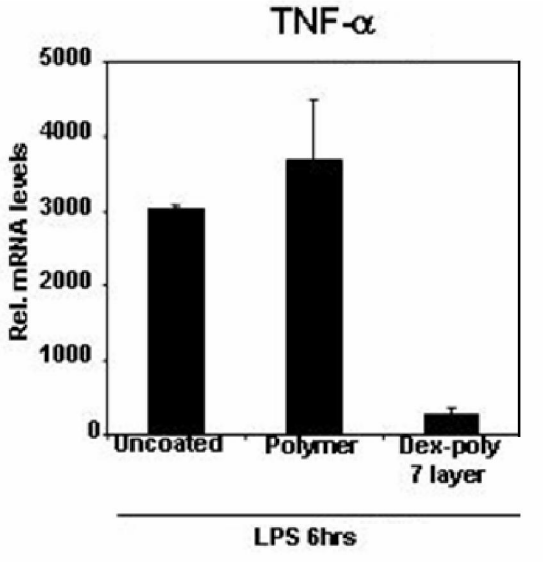

Figure 6. Dex-functionalized films show an early complete suppression of TNF production from RAW cells.

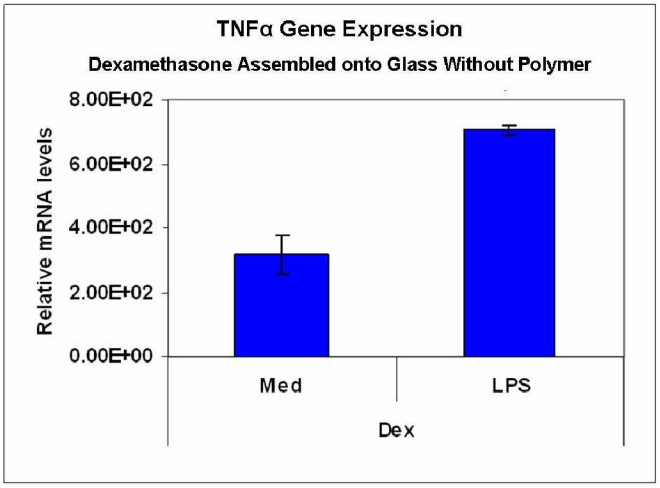

Figure 7. For samples where Dex was self-assembled onto the glass substrate *without* the polymer, it could be seen that stress attenuation was precluded. This showed that the copolymer enabled both interfacial Dex deposition as well as the ability to tether the Dex to the substrate and sustain the interface between the macrophages and anti-inflammation capabilities of the composite material.

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